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ORIGINAL PAPER

FAME Profiles in *Pseudomonas vesicularis* during Catechol and Phenol Degradation in the Presence of Glucose as an Additional Carbon Source

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Abstract

The aim of this study was to evaluate the impact of catechol and phenol added to culture media separately and with glucose as an additional, easily-degradable carbon source on fatty acid methyl ester (FAME) composition in *Pseudomonas vesicularis*. Simultaneously, the degradation rates of aromatic substrates used were investigated in single and binary substrate systems. Both catechol and phenol treatments caused changes in the distribution of tested groups of fatty acids. The most noticeable changes included an increase in degree of fatty acid saturation, the appearance of branched and disappearance of hydroxy fatty acids as compared to the control sample with glucose. Under catechol or phenol treatment sat/unsat ratio showed the values of 8.63 and 11.38, respectively, whereas in control cells it reached the value of 2.66. The high level of saturation comes from the high content of cyclopropane fatty acids in bacteria under exposure to aromatic substrates, regardless of the presence of glucose. In these treatments their content was more than 3-fold higher compared to the control. It has been demonstrated that glucose supplementation of culture media containing single aromatic substrate extended the degradation rates of catechol and phenol by *P. vesicularis*, caused an increase in number of cells but did not significantly change the fatty acid profiles in comparison with bacteria growing on catechol and phenol added to the media individually.

Key words: *Pseudomonas vesicularis*, catechol and phenol degradation, fatty acid composition

Introduction

Fatty acids are essential structural components of bacterial cell membranes that regulate their stability and fluidity. The membrane is the site of the primary contact with the environment and has an important role in maintaining the viability and functionality of bacterial cells. The main function of the membrane is to form permeability barriers regulating the passage of solutes between the cell and the external environment. This function is mainly determined by membrane lipid composition (Šajbidor, 1997; Denich *et al*., 2003). The analysis of bacterial membrane fatty acids is also of interest for studies on toxicity of many contaminants that generate environmental stress. Many findings documented that xenobiotics, such as organic solvents and aromatic hydrocarbons influence bacterial fatty acid composition (Heipieper *et al*., 1992; Sikkema *et al*., 1994; Kabelitz *et al*., 2003). Low-molecular weight aromatic hydrocarbons, such as catechol and phenol are the simplest structurally aromatic compounds and enter the environment as a consequence of human activities. For example, they widely occur during the production of dyes, pesticides, pharmaceuticals, wood processing chemicals, polymers and explosives. Since aromatic compounds exhibit toxic, mutagenic and carcinogenic properties, there is a serious concern about their elimination from environment. One of the most promising methods is the application of hydrocarbon degrading bacteria to clean-up contaminated sites. For this purpose, numerous bacteria mainly from the genera *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Bacillus* are used in the field of phenols degradation (Ahamad and Kunhi, 1996; Chang *et al*., 1997; Ali *et al.*, 1998; Heesche-Wagner *et al*., 1999; Beendorf *et al*., 2001).

The rate of degradation processes of many aromatic substrates can be improved by supplementing

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of culture media with additional carbon sources or other compounds such as nitrate, phosphate as well as mineral constituents. Glucose, sodium glutamate and yeast extracts are known as conventional carbon sources that influence the biotransformation and biodegradation processes (Wang *et al*., 1996; Wang and Loh, 1999, 2001). For example, Yu and Ward (1994) have observed that the rate of pentachlorophenol degradation by mixed bacteria cultures significantly increased by the addition of glucose and peptone to culture medium. However, some additional carbon sources may inhibit aromatic compound degradation. In studies conducted by Ampe *et al*. (1998) it has been shown that *Ralstonia eutropha* degraded phenol less effectively in the presence of acetate as compared to the culture with phenol alone. Moreover, glucose enrichment repressed catechol degradation by *Pseudomonas* sp. CF600 (Mrozik *et al*., 2006). In turn, the addition of glucose and sodium glutamate did not affect the dynamics of phenol degradation by *Pseudomonas putida* ATCC49451 (Loh and Wang, 1998). Bacteria posses a regulatory mechanism that allows them to use a preferential carbon source over a mixture of several other substrates and this phenomenon is usually called catabolic repression. It has been described for various bacteria, however, the molecular mechanism of gene expression for peripheral catabolic enzymes in the presence of preferred substrate differ substantially between species (Saier, 1996; Stülke and Hillen, 1999; Petruschka *et al.*, 2001).

In fact, many aromatic compounds partition into phospholipid bilayer and modify its fatty acids composition and membrane properties. Accumulation of these compounds in the membrane disturbs many biological processes such as respiration, growth, ions and nutrient transport and may even cause lysis of the cell (Sikkema *et al*., 1995; Weber and de Bont, 1996; Denich *et al*., 2003). As a response to phenols exposure bacteria modify their membrane lipid composition by *de novo* synthesis of fatty acids, isomerization of *cis* to *trans* unsaturated fatty acids, changing the proportion between *iso* and *anteiso* branched fatty acids, altering the average of chains length and protein content (Keweloh *et al*., 1990; Heipieper *et al*., 1994; Sikkema *et al*., 1995). These mechanisms have been related to homeoviscous adaptation and have been investigated by several authors (Shinitzky, 1984; Heipieper *et al*., 1992; Härtig *et al.*, 2005).

However, there is no available information on the influence of additional carbon sources on fatty acid profiles of bacteria during the biodegradation of phenols. The objective of this work was to establish changes in cellular fatty acid patterns in *Pseudomonas vesicularis* during catechol and phenol degradation in culture media supplemented with glucose as an additional source of carbon and energy.

Experimental

Materials and Methods

Bacterial strain. The experiments were performed using *Pseudomonas vesicularis* strain isolated from mixed populations of activated sludge collected from sewage-treatment plant in Częstochowa, Poland. To select phenol-degrading bacteria the increasing doses of phenol were added to sample of sludge for 30 days. To isolate phenol-degrading bacteria 10-fold dilutions of sludge suspensions were plated onto mineral medium (Kojima *et al*., 1961) amended with 0.188 g/l of phenol. Among isolated strains *P. vesicularis* was dominant. It was identified on the basis of cellular fatty acids derivatized to methyl esters (FAMEs) and analysed by gas chromatography using the MIDI Microbial Identification System (Newark, USA).

Culture conditions. Cultures were grown in modified minimal medium containing: 3.78 g of $Na_2HPO_4 \times 12H_2O$; 0.5 g of KH_2PO_4; 5.0 g of NH_4Cl; 0.2 g of $MgSO_4 \times 7H_2O$ and 0.1 g of yeast extract in 1.0 l of deionised water (Kojima *et al.*, 1961). To study the effect of glucose on FAME profiles bacteria were cultivated in Kojima medium containing catechol or phenol, at the concentration of 0.440 g/l and 0.376 g/l, respectively and in binary mixtures containing single aromatic substrate and 1.0 g/l of glucose. The final pH of the medium was 7.2–7.3. Liquid cultures were grown in 500 ml flask on rotary shaker (125 rpm) at 30°C.

Bacterial growth. Samples of the cultures were withdrawn every two hours until 8 h of incubation, and then at 16 and 24 h of the experiments. Cell density (OD) was measured spectrophotometrically as the absorbance of the suspension at 600 nm, with reference to a standard curve calibrated by plate enumeration.

Determination of catechol and phenol concentrations. Concentrations of tested aromatic compounds were measured at the same sampling time when OD was measured. Determination of catechol concentration was based on color reaction between catechol and sodium molybdate by measuring absorbance at 480 nm (Evans, 1946). Phenol concentration was estimated using spectrophotometry method with diazotised *p*-nitroaniline by measuring the absorbance of color solution at 550 nm (Lurie and Rybnikova, 1968).

Determination of glucose concentration. Removal of glucose in media was calculated using tests GLUCOSE EO produced by Biochemtest, Poland. This test is based on glucose oxidation to gluconic acid by glucose oxidase with production of hydrogen peroxide in the presence of peroxidase and chromogene ABTS. Absorbance of solution was measured with spectrophotometer at 675 nm.

Enzyme activity assay. The activities of catechol dioxygenases were measured spectrophotometrically

by monitoring the formation of the first product of aromatic ring cleavage, *cis*, *cis*-muconate at 260 nm for catechol 1,2-dioxygenase and 2-hydroxymuconic semialdehyde at 375 nm for catechol 2,3-dioxygenase (Feist and Hegeman, 1969). Detailed procedure of enzymes isolation was described in the previous paper (Mrozik *et al*., 2006). Enzymes activities were expressed as mmol of *cis*, *cis*-muconate and 2-hydroxymuconic semialdehyde formed per mg of protein per minute for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively. The protein content of cell-free extract was estimated by the method of Bradford (1976) with lysozyme as a standard.

Fatty acid extraction and analysis. Fatty acid composition of bacterial strain was determined at mid-exponential phase of culture growth. For analysis of cellular fatty acids cells grown in single- and binary-substrates systems were used. Bacteria were harvested by centrifugation (8000×g) at 4°C for 30 min. The cell pellets obtained from each culture were washed with 10.0 ml of 0.85% NaCl to remove residue of culture medium. To decrease the humidity of bacterial cell, pellets were left through 2 h at room temperature. Next 40 mg of bacterial biomass was transferred in triplicate to reaction tubes (Pyrex). To each sample 1.0 ml of 3.27 M NaOH in MeOH:H_2O (1:1) for saponification was added. Then the samples were vortexed and placed in 100°C water bath for 30 min. Following this saponification step, fatty acids were converted to fatty acid methyl esters (FAMEs) by adding 2.0 ml of 6.0 M HCl:MeOH (1:0.85) to each tube and were incubated at 80°C in water bath for 10 min. FAMEs were extracted from the aqueous phase by addition of 1.15 ml of hexane:methyl tert-butyl ether (MTBE) (1:1) to each tube. Then samples were rotated end-over-end for 10 min. After removing aqueous (lower) phase, 3.0 ml of 0.3 M NaOH in H_2O was added and the tubes were again rotated for 5 min (Sasser, 1990). Finally, the organic (upper) phases containing FAMEs were transferred to gas chromatography vials. Fatty acids were analysed by gas chromatography (Hewlett-Packard 6890, USA) using capillary column Ultra 2-HP (cross-linked 5% phenyl-methyl silicone 25 m, 0.22 mm ID, thickness 0.33 mm) and hydrogen as a carrier gas. FAMEs were detected by a flame ionisation detector (FID) and identified by MIS (Microbial Identification System) software, using the aerobe TSBA40 method and TSBA40 library (MIDI, USA).

Results and Discussion

Cell growth and aromatic compounds degradation. To estimate the effect of glucose on catechol and phenol degradation by *P. vesicularis* bacteria were grown in media containing only single aromatic substrate and in the same media supplemented with glucose. The strain was able to metabolize catechol completely at the concentration of 0.440 g/l and phenol at the concentration of 0.376 g/l served as a single substrate during 10 and 15 h, respectively. The highest catechol removal was observed during the first 4 h of culturing and in this time 65% of dose added to the medium was degraded. In comparison, in that time in phenol containing medium its concentration decreased about 42%. In both experiments significant differences in growth as indicated OD value of *P. vesicularis* were not found. The substrate removal profiles and growth curves are presented in Figure 1A and B.

The addition of glucose to media with aromatic substrates resulted in the increase of culture OD and altered the time of catechol and phenol degradation. The time necessary for complete removal of both aromatic substrates by tested bacteria extended to 24 h (Fig. 1A and B). In comparison with single-substrate system, OD of bacterial culture in binary mixtures was markedly higher and reached the value of 0.8 and 1.0 for catechol with glucose and phenol with glucose, respectively. In control medium with glucose served as a sole source of carbon and energy *P. vesicularis* metabolized it during 6 h of culturing (data not shown). Time of glucose utilization did not change in the medium containing phenol whereas in medium with catechol was 2 hours shorter as compared to the control sample. Interesting changes were revealed when compared the dynamics of catechol and phenol biodegradation. In mixture containing glucose and phenol both substrates started to be degraded at the same time whereas in the mixture containing catechol and glucose were not degraded simultaneously (Fig. 1A and B). Catechol biodegradation by *P. vesicularis* started when 90% of glucose added was metabolized. These results indicated that glucose was preferentially utilized by *P. vesicularis* and it might repress catechol degradation. Similar phenomenon was observed during studies on catechol biodegradation rate in the presence of glucose by strain *Pseudomonas* sp. CF600. The time of catechol degradation in binary system with glucose was longer than that when catechol served as a sole carbon source. In contrast to *P. vesicularis* it started to degrade both substrates immediately after their addition to the culture medium (Mrozik *et al*., 2006). The effect of glucose and sodium acetate on aromatic compounds biodegradation by bacteria from the genus *Pseudomonas* was also observed by Kao *et al*. (2005). They have revealed that addition of these extra carbon sources did not enhance pentachlorophenol (PCB) degradation by *Pseudomonas mendocina* NSYSU. They have explained this phenomenon by the fact that this strain isolated from PCB-contaminated soil did not receive inputs of glucose and acetate from natural sources and the cometabolism is not the

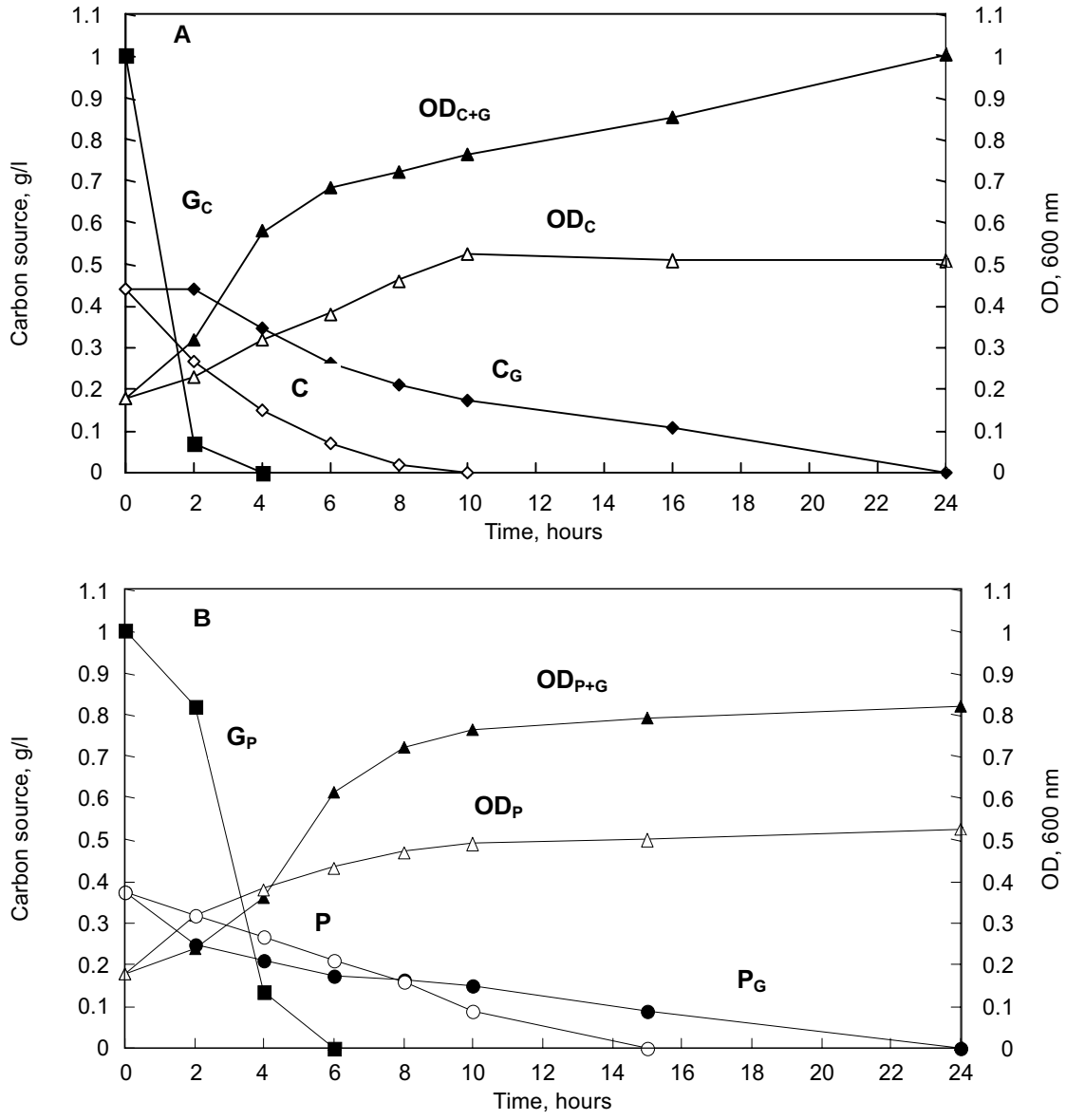

Fig. 1. Degradation rate of catechol (0.440 g/l) (A) and phenol (3.376 g/l) (B) in the presence and absence of glucose (1.0 g/l) and growth curves of *Pseudomonoas vesicularis*.

C – degradation of catechol, G_C – degradation of glucose in binary system with catechol, C_G – degradation of catechol in binary system with glucose, OD_C – optical density of culture growing on catechol, OD_{C+G} – optical density of culture growing on catechol and glucose, P – degradation of phenol, G_P – degradation of glucose in binary system with phenol, P_G – degradation of phenol in binary system with glucose, OD_P – optical density of culture growing on phenol, OD_{P+G} – optical density of culture growing on phenol and glucose.

dominant biodegradation mechanism of PCB by this bacterium. In turn, impact of glucose on phenanthrene (PHE) degradation by *Sphingomonas* sp. strain LB126 in chemostat cultures was studied by van Herwijnen *et al*. (2003). They found that PHE removal in the presence of glucose was much higher as compared to phenanthrene and fluorene grown culture without the high impact on growth cells. Besides biodegradation experiments many studies are being conducted on molecular mechanism of catabolic repression in bacteria grown on glucose or other easily-degradable carbon sources and aromatic compounds as inducers. For example, Duetz *et al*. (1996) described catabolic repression of the TOL pathway by succinate under different conditions of inorganic-nutrient limitation. In other studies the role of Crc regulator in the repression of several catabolic pathways for the assimilation of some sugars and aromatic compounds in *Pseudomonas putida* was shown (Morales *et al*., 2004). Such studies are necessary for better understanding the correlations among degradation ratio of various organic substrates by bacteria.

In parallel to biodegradation studies the activities of enzymes involved in aromatic ring cleavage were calculated. As shown in Table I, *P. vesicularis* in each experiment treatment synthesized both catechol 1,2- and 2,3-dioxygenases. In bacterial cells growing on catechol only the activity of catechol 1,2-dioxygenase

Table I
Catechol 1,2- and 2,3-dioxygenase activities in cell-free extracts of *Pseudomonas vesicularis* growing on catechol or phenol with/without glucose

Carbon source	Catechol 1,2-dioxygenase µmol/min/mg of protein	Catechol 2,3-dioxygenase µmol/min/mg of protein
glucose	0.07 ± 0.02	0.11 ± 0.03
catechol	2.55 ± 0.07	0.39 ± 0.07
catechol + glucose	2.11 ± 0.11	0.19 ± 0.05
phenol	0.70 ± 0.05	1.85 ± 0.17
phenol + glucose	0.57 ± 0.06	1.12 ± 0.09

Number of replicates, n = 3

Table II
Percentages of total saturated, unsaturated fatty acids and sat/unsat ratio of *Pseudomonas vesicularis* growing on catechol or phenol, or/and glucose in single- and binary systems

Group of fatty acids	% of total fatty acids				
	Glucose	Catechol	Catechol + glucose	Phenol	Phenol + glucose
Saturated	72.65	89.41	87.00	91.92	84.27
Straight-chain	55.40	50.99	48.39	50.52	52.58
Hydroxy	5.70	0.00	0.00	0.00	0.00
Branched	0.00	0.93	0.84	1.62	1.12
Cyclopropane	11.56	37.49	37.77	39.78	30.57
Unsaturated	27.35	10.36	12.99	8.08	15.73
Sat/unsat ratio	2.66	8.63	6.70	11.38	5.36

Values are the averages of three independently performed experiments (standard errors < 5%).

was significantly higher as compared to activity of catechol 1,2-dioxygenase and reached the values of 2.55 and 0.39 µmol/min/mg of protein, respectively. In contrast, in bacteria cultured on phenol the activity of catechol 2,3-dioxygenase was 2.5-fold higher than catechol 1,2-dioxygenase. These results indicate that catechol and phenol degradation proceeded both *via meta* and *ortho* metabolic pathways. The addition of glucose slightly decreased the activity of measured enzymes (Table I). Similarly, Tian *et al*. (2003) studying the impact of glucose added on phenanthrene degradation by *P. mendocina* demonstrated that glucose supplementation decreased the activities of hydrocarbon dioxygenase and catechol 2,3-dioxygenase.

FAME analysis. To estimate the changes in fatty acid composition the profiles of whole-cell fatty acids isolated from *P. vesicularis* cultured in media containing catechol or phenol degradation with or without glucose were analyzed. For the detailed interpretation of results all fatty acids obtained were divided into two major groups: saturated and unsaturated. The first group of fatty acids included four sub-groups: straight-chain, branched, hydroxy- and cyclopropane fatty acids. Percentages of these fatty acid groups in each experiment treatments are presented in Table II. Both catechol and phenol treatment caused crucial changes in the distribution of the tested groups of whole cell-derived fatty acids in *P. vesicularis*. Bacteria cultured on aromatic substrates characterized by the higher proportion of saturated fatty acids as compared to control with glucose. The percentage of these fatty acids composed 89.41% and 91.92% of total fatty acids when bacteria were grown on catechol or phenol, respectively, whereas in control sample they represented 72.65% of total fatty acids (Table II). Similar tendency resulting in the increase of the membrane saturation in the presence of toxic aromatic compounds and aliphatic alcohols was earlier observed in studies using *P. putida* (Heipieper *et al*., 1992; Mrozik *et al*., 2005), *Rhodococcus* sp. 33 (Gutierrez *et al.*, 1999), *Ralstonia eutropha* H850 (Kim *et al*., 2001) and *Acinetobacter calcoaceticus* (Kabelitz *et al*., 2003). The addition of glucose to culture medium with catechol did not significant change the abundance of saturated fatty acids in *P. vesicularis* as compared to cells collected from medium containing catechol only. In contrast, in bacteria growing in medium containing phenol and glucose the proportion of saturated fatty acids was about 10% lower than in bacteria grown on phenol used separately. In bacterial cells, irrespective of medium content, among saturated fatty acids the dominant group was straight-chain fatty acids. This group included the following fatty acids: 10:0, 12:0, 14:0, 15:0, 16:0, 18:0 and 19:0. However, their percentages in fatty acid profiling obtained from bacteria cultured in single- and binary system was lower as compared to control (55.40%) and ranged from 48.39 to 52.58%. The increase of degree of membrane saturation is well known adaptive mechanism allowing bacteria to survive under toxic substrates stress (Sikkema *et al*., 1994, 1995; Weber and de Bont, 1996). Catechol and phenol exposure drastically changed the content of terminally branched and hydroxy fatty acids. Interestingly, this observed effect was independent of the presence of glucose in the culture medium. The results showed that both aromatic substrates caused the disappearance of hydroxy fatty acid 12:0 2OH, whereas in control sample with glucose it composed 5.70% of total fatty acids. In contrast, catechol and phenol in all tested systems caused the appearance of branched fatty acid 15:0 *iso* and additionally 15:0 *anteiso* was detected in cells growing on phenol as a single carbon source. However, the percentages of branched fatty acids in FAME profiles were generally low and ranged from 0.84 to 1.62% of total fatty acids for bacteria grown on catechol and/or phenol in the presence or absence of glucose (Fig. 2). Tsitko *et al.* (1999) studying the impact of different aromatic compounds on

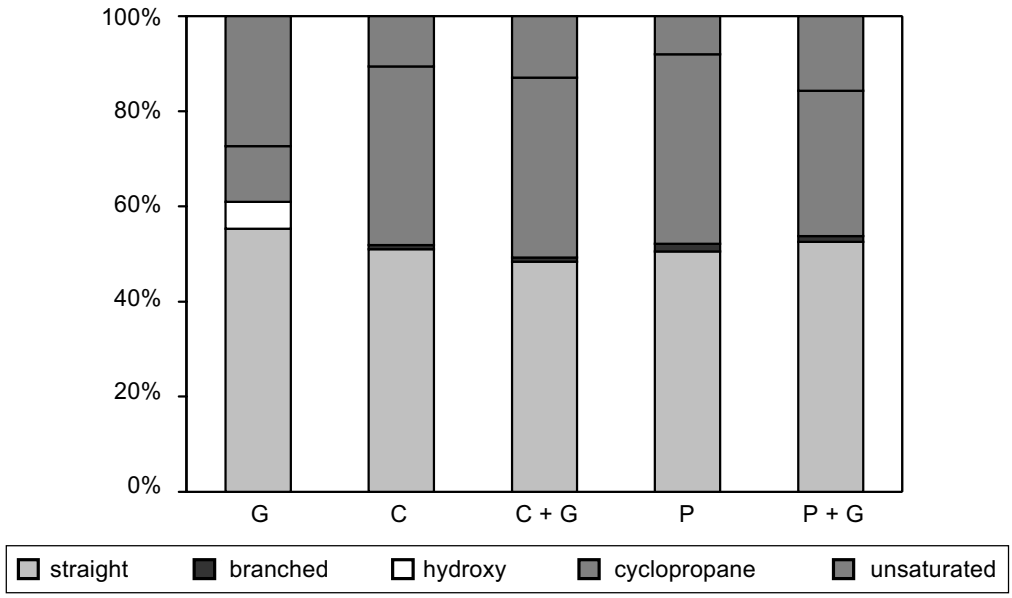

Fig. 2. Percentages of distinct groups of fatty acids in *Pseudomonas vesicularis* growing on catechol or phenol only and in binary systems with glucose, G – glucose, C – catechol, P – phenol

R. opacus FAMEs composition revealed that these substrates also increased content of branched fatty acids.

It seems that the response of bacterial cells to membrane active substrates to a large extent depends on individual physiological and biochemical features of given bacteria. In contrast to tested strain, *Pseudomonas* sp. CF600 reacted to catechol and phenol exposure in an opposite way. This strain growing on catechol or phenol in single- and binary systems with glucose synthesized both hydroxy and branched fatty acids (Mrozik *et al.*, 2006). With regards to the chromatographic profiles of saturated fatty acids, the changes in the abundance of cyclopropane fatty acids 17:0 *cy* and 19:0 *cy* ω8*c* were the most visible. The highest increase of these fatty acids content was detected in bacterial cells grown on phenol. Their percentage reached the value of 39.78% whereas in control sample with glucose showed the value of 11.56%. Surprisingly, in bacteria cultured in binary system containing phenol and glucose 19:0 ω8*cy* fatty acid was not detected that resulted in decreasing of the total amount of cyclopropane fatty acids. Such phenomenon was not observed in the experiment with catechol served as a sole carbon and energy source and in mixture with glucose (Fig. 2). The presented results as well as results obtained by other researchers indicate that content of cyclopropane fatty acids depends not only on the chemical structure and properties of hydrocarbons but also on the features of bacterial strains (Ramos *et al*., 1997; Kim *et al*., 2001; Fang *et al.*, 2004; Mrozik *et al*., 2006). Cyclopropane fatty acids have been known as compounds that stabilize membrane lipids, make it more rigid and in this way improve bacteria survival under unfavorable conditions. However, the detailed role of these fatty acids in the regulation of bacterial membrane stability and fluidity in the presence of aromatic compounds is not fully understood yet and require further investigations and explanations.

It has been found that tested aromatic substrates used both in a single- and binary systems significantly decreased the amount of unsaturated fatty acids such as 16:1ω7*c* and 18:1ω9*c*. Their abundance declined about 5-fold in comparison to control. In contrast, the percentage of fatty acid 18:1ω7*c*/ω9*t*/ω12*t*, which is well known as typical for bacteria from the genus *Pseudomonas*, increased from 6.34% in control to 8.41% and 10.25% in bacterial cells growing in media supplemented with catechol or phenol and glucose, respectively. However, under exposure of catechol and phenol used individually the amount of this fatty acid slightly decreased.

The impact of various toxic compounds and their interactions with easily degradable carbon sources on bacterial MIDI-FAME profiles might be examined by analysis of saturated/unsaturated ratio (Table II). In this study it has been showed that under catechol or phenol exposure this ratio was about 3.5-fold higher than that in control and reached the value of 8.63 and 11.38 for catechol and phenol, respectively. The addition of glucose to culture medium containing the aromatic substrates changed the response of bacterial cells to these toxic compounds which resulted in decreasing of sat/unsat ratio in comparison with cultures grown in the presence of catechol or phenol separately (Table II). Additionally, the protective effect of glucose against the toxicity of aromatic substrates during their biodegradation was confirmed by marked increase of bacterial culture density (Fig. 1A and B).

The action of catechol or phenol in the presence of glucose as an additional carbon source measured as FAMEs patterns of bacterial cells was slightly different as compared to the action of these aromatic substrates added individually. Generally, the differences among the fatty acid composition in bacteria cultured in media containing catechol or phenol and in mixtures with glucose were slight but significant in comparison to pattern of FAMEs obtained for control samples. The most noticeable difference was associated with cyclopropane fatty acid abundance. Beside straight-chain fatty acids they constituted the second dominant group in FAMEs profiles. The high proportion of cyclopropane fatty acids resulted in the increase of sat/unsat ratio. The data obtained from biodegradation studies and analysis of FAME profiles of *P. vesicularis* indicated that the addition of glucose as easily-degradable carbon source to media containing aromatic substrates such as catechol and phenol stimulated the growth of bacteria while did not have distinct influence of the whole cell-derived fatty acid composition.

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